

# Stress-Induced Outer Membrane Vesicle Production by *Pseudomonas aeruginosa*

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As an opportunistic Gram-negative pathogen, *Pseudomonas aeruginosa* must be able to adapt and survive changes and stressors in its environment during the course of infection. To aid survival in the hostile host environment, *P. aeruginosa* has evolved defense mechanisms, including the production of an exopolysaccharide capsule and the secretion of a myriad of degradative proteases and lipases. The production of outer membrane-derived vesicles (OMVs) serves as a secretion mechanism for virulence factors as well as a general bacterial response to envelope-acting stressors. This study investigated the effect of sublethal physiological stressors on OMV production by *P. aeruginosa* and whether the *Pseudomonas* quinolone signal (PQS) and the MucD periplasmic protease are critical mechanistic factors in this response. Exposure to some environmental stressors was determined to increase the level of OMV production as well as the activity of AlgU, the sigma factor that controls MucD expression. Overexpression of AlgU was shown to be sufficient to induce OMV production; however, stress-induced OMV production was not dependent on activation of AlgU, since stress caused increased vesiculation in strains lacking *algU*. We further determined that MucD levels were not an indicator of OMV production under acute stress, and PQS was not required for OMV production under stress or unstressed conditions. Finally, an investigation of the response of *P. aeruginosa* to oxidative stress revealed that peroxide-induced OMV production requires the presence of B-band but not A-band lipopolysaccharide. Together, these results demonstrate that distinct mechanisms exist for stress-induced OMV production in *P. aeruginosa*.

Bacterial pathogens must respond to changes and challenges in their environment in order to survive and establish an infection. Bacterial virulence factors such as adhesins, toxins, and enzymes contribute by providing secreted defenses against antibacterial factors, as well by generating nutrients for the bacteria. To counteract environmental effects that compromise their structural integrity, bacteria mount stress responses that typically consist of the activation of a stress sensor, changes in the transcriptional profile, and downstream changes to the bacterial envelope composition (1). Outer membrane vesicles (OMVs) produced by pathogens can serve as secretion vehicles for complexes of inflammatory and virulence-associated factors (2, 3). Intriguingly, OMVs have also been identified as a stress response that can rid bacteria of misfolded protein as well as provide an external decoy to absorb cell surface-acting antimicrobial agents (3, 4). These offensive and defensive properties demonstrate that OMV production could serve multiple roles in bacterial pathogenesis.

OMVs are 50- to 250-nm spherical bodies composed of a subset of outer membrane components (lipopolysaccharide [LPS], phospholipids, and integral membrane proteins), as well as soluble proteins that originate mainly from the periplasm (3, 5–7). The selective composition of OMVs and the lack of concurrent cell lysis during their formation suggest that OMV production is a regulated secretion mechanism (6–14). OMV production has been observed in biopsies of tissues infected with *Shigella dysenteriae*, enterotoxigenic *Escherichia coli*, *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Pseudomonas aeruginosa* (2, 15). The small size of OMVs likely facilitates dissemination into tissues further than the bacterium can reach, and the membrane component offers protection from host proteases, allowing the delivery of these bacterial products distal to the site of colonization.

Opportunistic pathogens such as *P. aeruginosa* encounter a variety of environmental changes and toxic stressors during infection as the organism moves from the environment into a host. *P.*

*aeruginosa* is a common cause of nosocomial acquired infections and a leading cause of morbidity and mortality in patients suffering from the genetic disease cystic fibrosis (16–19). To counteract host stressors, this Gram-negative pathogen produces several secreted virulence factors, including degradative enzymes (proteases, lipases) and redox compounds (pyocyanin) (5, 13, 20, 21). However, most of what is known about *P. aeruginosa* stress response pathways centers around the production of the exopolysaccharide capsule, which is composed primarily of alginate. Alginate protects the bacterium from phagocytosis, oxidative stress, and complement (22–25).

OMVs are proposed to aid *P. aeruginosa* pathogenesis by contributing to a variety of virulence-associated processes. *P. aeruginosa* virulence factors are associated with OMVs, including peptidoglycan hydrolase, phospholipase C, hemolysin, esterase, metalloprotease, and alkaline phosphatase (13, 14, 20, 21, 26). Host cells can internalize *P. aeruginosa* OMVs, leading to host cell death by disruption of the electron transport chain and trafficking pathways (9, 27–30). Further, *P. aeruginosa* LPS presented to macrophages in the context of OMVs is a potent inducer of cytokines that can generate a destructive inflammatory response (31). Because *P. aeruginosa* OMVs can kill both Gram-negative and Gram-positive bacteria in cocultures, OMVs are also impli-

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cated in facilitating the removal of competing bacteria from the environment during an infection (26, 32).

The dynamics of OMV formation is not well understood despite the ubiquitous production of OMVs by Gram-negative bacteria; however, some mechanistic concepts have begun to emerge. Mutants lacking particular outer membrane anchoring lipoproteins exhibit high levels of OMV budding, suggesting that OMV production requires regulated, local depletion or removal of such linkages by an as-yet-undefined mechanism (3, 33–35). Additionally, for *P. aeruginosa*, the multifunctional *Pseudomonas* quinolone signaling (PQS) molecule 2-heptyl-3-hydroxy-4-quinolone has been proposed as a key reagent responsible for OMV formation (36). The addition of PQS to pure LPS facilitates the formation of LPS micelles, and PQS can bind and intercalate into the bacterial outer membrane (37, 38). The accumulation of negative charges by the addition of PQS to LPS is thought to create repulsion and, thereby, a force sufficient to bend the membrane, ultimately leading to vesicle budding (37). Consistent with a repulsion-mediated membrane-bending effect, native OMVs were also found to be highly enriched in LPS with the longer, highly charged form of O antigen (B-band LPS), compared to the *P. aeruginosa* outer membrane, which contains both short, uncharged (A-band) and B-band LPS (13, 20).

A role for OMVs as an envelope stress response mechanism was first elucidated in *E. coli*. Mutations in several  $\sigma^E$  envelope stress response pathway genes (*rseA*, *degP*, and *degS*) were discovered to cause constitutively high levels of OMVs (8). The *E. coli*  $\sigma^E$  pathway responds to the presence of misfolded outer membrane proteins located in the periplasmic space by triggering a proteolytic cascade resulting in the activation of the alternative sigma factor  $\sigma^E$  (encoded by *rpoE*) and the consequent upregulation of the extensive  $\sigma^E$  regulon (39, 40). It was further demonstrated that a fusion protein mimicking an unfolded outer membrane protein that activated  $\sigma^E$  was 10-fold enriched in OMVs compared with other periplasmic proteins (12). Thus, OMVs were proposed to remove unfolded proteins from the periplasmic space as an independent envelope stress response. Mutants of *E. coli* that constitutively undervesiculate are hypersensitive to external stressors, demonstrating that OMVs are a critical bacterial stress response (12).

*P. aeruginosa* contains a homologue of the *E. coli*  $\sigma^E$  envelope stress pathway, including the alternative sigma factor AlgU (also named AlgT) and the periplasmic protease/chaperone MucD, although this pathway is not as well characterized (22–25). In addition to having a potential role as a mediator of envelope stress homologous to RpoE in *E. coli*, AlgU has been well studied for its role in activating alginate biosynthesis. Alginate is used by *P. aeruginosa* as a primary defense mechanism against host stressors and has been associated with biofilm formation (25). Unlike *rpoE* in *E. coli*, *algU* is not essential for growth (41). Also, RpoE in *E. coli* is thought to be almost entirely sequestered by the anti-sigma factor RseA, whereas 33% of AlgU in *P. aeruginosa* exists free in the cytoplasm despite the presence of a homologous anti-sigma factor, MucA (42). Recently, Tashiro et al. studied the effect of an *algU* deletion on vesiculation by *P. aeruginosa* (43). Their results indicated that the absence of AlgU resulted in an increase in OMV production. It was hypothesized that without AlgU, the bacteria could not cope with the normal rate of misfolding of outer membrane proteins, resulting in envelope stress and an increase in OMV production.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
<i>E. coli</i> strains		
DH5 $\alpha$	Cloning strain	Gibco
DH10 $\beta$	Cloning strain	Gibco
Ec233	<i>E. coli</i> /pMT616, Cm <sup>r</sup>	61
Ec822	<i>E. coli</i> /pLW112, Amp <sup>r</sup>	23
Ec869	<i>E. coli</i> /pLW127, Amp <sup>r</sup>	24
Ec870	<i>E. coli</i> /pMF54, Amp <sup>r</sup>	62
Ec887	<i>E. coli</i> /pIM001, Amp <sup>r</sup>	This work
<i>P. aeruginosa</i> strains		
Pa725	Strain PAO1	24
Pa866	Strain PA14	41
Pa867	PA14 $\Delta$ algU, Gm <sup>r</sup>	41
Pa872	PA14/pLW127, Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa874	PA14/pMF54 Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa876	PA14 $\Delta$ algU/pMF54, Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa877	PA14 $\Delta$ algU/pIM001, Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa879	PA14 $\Delta$ pqsA, Gm <sup>r</sup>	41
Pa891	PA14/pLW112, Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa892	PA14/pIM001, Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa1300	PA14 $\Delta$ algD/pMF54, Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa1301	PA14 $\Delta$ algD/pIM001, Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Pa1302	PA14 $\Delta$ wbpM, Gm <sup>r</sup>	41
Pa1303	PA14 $\Delta$ wapR, Gm <sup>r</sup>	41
Pa1304	PA14 $\Delta$ rmd, Gm <sup>r</sup>	41
Pa1305	PA14 $\Delta$ algD, Gm <sup>r</sup>	41
Pa1312	PAO1/pLW112, Gm <sup>r</sup> Cb <sup>r</sup> Amp <sup>r</sup>	This work
Plasmids		
pMT616	Triparental mating helper plasmid, Cm <sup>r</sup>	61
pLW127	pOsmC::LacZ, AlgU-dependent promoter fused to LacZ, Amp <sup>r</sup>	24
pMF54	pMF54 expression vector, Amp <sup>r</sup>	62
pLW112	pMF54-based MucD expression plasmid, Amp <sup>r</sup>	23
pIM001	pMF54-based expression vector for AlgU, Amp <sup>r</sup>	This work

The focus of this study was to determine whether *P. aeruginosa* increased OMV production in response to stress and, if so, whether modulation of OMVs was based on previously identified regulatory mechanisms. Stress-induced OMV production by *P. aeruginosa* would further support the hypothesis that *P. aeruginosa* OMVs contribute to disease, as the host environment is very hostile. We used the peptidoglycan synthesis inhibitor D-cycloserine, and three physiologically relevant stressors (hydrogen peroxide, polymyxin B, and temperature) to assess OMV abundance, and we investigated whether AlgU, MucD, and PQS played a role in these stress-regulated levels of OMVs. We concluded that none of the identified mechanisms held for all stressors tested, and we discovered that B-band LPS is required for induced OMV formation upon oxidative stress. The characterization of stress-induced OMV production by pathogens begins to provide insight into how OMVs could benefit bacterial infection and colonization.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PA14 (wild type), PA14  $\Delta$ algU, PA14  $\Delta$ algD, PA14  $\Delta$ pqsA, PA14  $\Delta$ wbpM, PA14  $\Delta$ wapR, and PA14  $\Delta$ rmd were obtained from the transposon insertion mutant collection at the

Broad Institute (University of Massachusetts) (41). The AlgU reporter plasmid pLW127 containing the AlgU-dependent OsmC promoter fused to  $\beta$ -galactosidase was generously provided by the Ohman Lab (24). pLW127 was transferred into the appropriate background by triparental mating using the pMT616 helper plasmid (provided by C. R. H. Raetz). PA14 mutant strains, PA14/pLW127, and *E. coli*/pMT616 were grown at 37°C in Luria-Bertani (LB) broth containing gentamicin (25  $\mu$ g/ml), carbenicillin (100  $\mu$ g/ml), and chloramphenicol (25  $\mu$ g/ml). Selection was done by plating on pseudomonas isolation agar (BD) containing carbenicillin. PA14/pIM001 was created by triparental mating between *E. coli*/pIM001, PA14, and *E. coli*/pMT616. Brain heart infusion (BHI) broth (37 g/liter) was used, as indicated in the legend for Fig. 5, for overnight PA14  $\Delta$ pqsA studies.

**Stress treatments.** Overnight cultures were used to inoculate 250 ml of LB broth at a 1:50 dilution and grown at 37°C with shaking (200 rpm) to mid-log phase (optical density at 600 nm [OD<sub>600</sub>], ~0.4). Cells were then pelleted (10,000  $\times$  g, 10 min) and resuspended in fresh 37°C LB medium to a final volume of 250 ml. Hydrogen peroxide, D-cycloserine, and polymyxin B were added to final concentrations of 250 to 1,000  $\mu$ M, 250  $\mu$ g/ml, and 4  $\mu$ g/ml, respectively. No stressor was added to negative-control cultures. Fresh hydrogen peroxide was added every hour to counteract peroxide degradation. Cultures were grown to an OD<sub>600</sub> of ~0.9 to 1.1. A portion of the culture was assessed for growth on agar plates to determine CFU (CFU/ml).

For temperature stress assays, bacterial cultures were inoculated as described above and grown overnight at 25°C until mid-log phase. Cells were pelleted, resuspended in fresh, prewarmed (25, 37, or 39°C) LB medium, and grown to an OD<sub>600</sub> of ~0.9 to 1.1. A portion of the culture was assessed for growth on agar plates to determine CFU/ml.

**Periplasmic MucD levels.** PA14/pLW127 was grown at 37°C in 250 ml of LB broth containing 100  $\mu$ g/ml carbenicillin with shaking (200 rpm) to mid-log phase (OD<sub>600</sub> ~ 0.4). Cells were then pelleted (10,000  $\times$  g, 10 min) and resuspended in fresh 37°C LB medium to a final volume of 250 ml. Hydrogen peroxide, D-cycloserine, and polymyxin B were added to final concentrations of 250  $\mu$ M, 250  $\mu$ g/ml, and 4  $\mu$ g/ml, respectively. Cells were pelleted, and periplasmic protein was isolated 15 to 30 min after treatment by osmotic and temperature shock, as follows. The cell pellet was resuspended in periplasmic shock buffer (50 mM Tris-Cl, 200 mM MgCl<sub>2</sub> [pH 7.3]) with alternating incubations at 37°C and 4°C (44). Cells were repelleted and the supernatant containing periplasmic contents were filtered through a 0.22- $\mu$ m syringe filter. Protein concentrations were determined by Bradford assay. MucD levels were determined by quantitative immunoblotting using anti-MucD antibody (kindly provided by D. Ohman). Due to multiple processed forms of MucD, densitometry of the entire lane was used to determine the total MucD present in the MucD immunoblots.

**OMV quantification.** Upon reaching an OD<sub>600</sub> of ~0.9 to 1.1 under the indicated conditions, the cultures were centrifuged (10,000  $\times$  g, 10 min), and the supernatant was filter sterilized through a 0.45- $\mu$ m membrane to remove any remaining cell debris. OMVs in the cell-free supernatants were then pelleted by centrifugation (38,000  $\times$  g,  $\geq$ 1 h) (45). The OMVs were resuspended in sterile phosphate-buffered saline (PBS) and filtered again through a 0.45- $\mu$ m filter. Samples were then concentrated by pelleting (100,000  $\times$  g, 1 h) and resuspended in 100  $\mu$ l sterile PBS. The quantity of OMVs in the resuspended material was assessed using the fluorescent lipophilic dye FM4-64 (Molecular Probes). Fluorescence was measured at 506 nm (excitation) and 750 nm (emission) to obtain relative fluorescence units/ml (RFU/ml). The number of RFU/ml was then divided by the number of CFU/ml determined at the time of vesicle harvest to generate the OMV yield (RFU/CFU). The RFU/CFU of OMV preparations from treated cultures was then divided by the RFU/CFU of OMV preparations from untreated cultures to determine fold OMV induction.

Since small amounts of OMVs were expected for PA14  $\Delta$ pqsA, a different method that better concentrates the OMVs was used to compare wild-type (WT) and  $\Delta$ pqsA OMV production. Late-log-phase culture

cell-free supernatants were precipitated using 71 to 75% ammonium sulfate at 4°C and floated in Optiprep gradients (45%, 40%, 35%, 30%, and 20% [wt/wt] Optiprep in 10 mM HEPES, 0.85% NaCl [pH 7.4]) as described previously (30). A portion of each fraction was analyzed by SDS-PAGE to identify the fractions containing outer membrane proteins. These OMV-containing fractions were combined, diluted, pelleted, and resuspended in sterile PBS. Comparative OMV yield was then determined using FM4-64 as described above and by densitometry of proteins detected by SDS-PAGE and ruby staining as previously described (9).

For experiments comparing OMV yield in stationary-phase cultures of PA14 and PA14 $\Delta$ pqsA using the protocol described in reference 36, 25 ml of BHI broth were inoculated with PA14 or PA14  $\Delta$ pqsA and grown 16 to 18 h. Cells were pelleted at 10,000  $\times$  g for 10 min, the supernatant was filtered through a 0.45- $\mu$ m membrane, cell-free supernatant was ultracentrifuged (70 min, 227,000  $\times$  g), and the pellet was resuspended in sterile PBS and refiltered through a 0.45- $\mu$ m membrane filter. Pelleted material was then quantitated using FM4-64 as described above or by measuring the absorbance at 220 nm as described previously (36), normalized to CFU at the time of harvest.

**AlgU reporter assay (Miller assay).** To determine the activity of AlgU, 25 to 50  $\mu$ l of each culture containing the pLW127 vector were lysed in a buffered solution containing 0.1% SDS and chloroform. The assay reaction was quenched by adding 1 M Na<sub>2</sub>CO<sub>3</sub>. AlgU reporter activity was quantified by measuring the absorbance at 420 nm and 550 nm of the samples in a solution containing 4 mg/ml of the chromogenic substrate ONPG (*ortho*-nitrophenyl- $\beta$ -galactoside). Each sample was measured in triplicate to account for assay error, and the values were normalized to CFU/ml and assay duration in minutes.

**AlgU overexpression.** AlgU was amplified from PA14 genomic DNA by PCR and added restriction endonuclease sites for (XbaI and XhoI) using the forward primer (5'-AATCTAGAATGCTAACCCAGGAACAG GATCA-3') and the reverse primer (5'-ATCTCGAGTCAGGCTTCTCG CAACAAAGGCT-3'). The AlgU fragment was then ligated into the pMF54 expression vector provided by the Ohman lab to create pIM001. pIM001 was then transformed into *E. coli* DH10 $\beta$  by electroporation and grown on LB ampicillin plates to select for transformants. The pIM001 plasmid was then moved into both the PA14  $\Delta$ algD and PA14  $\Delta$ algU backgrounds by triparental mating. AlgU expression was induced with 200  $\mu$ M IPTG at mid-log-phase growth. AlgU induction was assessed by immunoblotting with the cross-reactive rabbit anti-RpoE primary antibody (kindly provided by C. Gross). To determine relative fold AlgU induction, equal volumes of treated and untreated cultures were separated by SDS-PAGE and immunoblotted, and the intensities of the immunoreactive AlgU bands in the samples were compared by densitometry.

**MucD overexpression.** PA14 or PAO1 containing pMF54 (vector) or pLW112 (MucD) was grown at 37°C in 250 ml of LB broth containing 100  $\mu$ g/ml carbenicillin with shaking (200 rpm) to mid-log phase (OD<sub>600</sub> ~0.4). Cells were then pelleted (10,000  $\times$  g, 10 min) and resuspended in fresh 37°C LB medium to a final volume of 250 ml. Cultures were induced with 1 mM IPTG and, as noted in the legends for Fig. 3 and 4, 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> was added (and supplemented hourly as indicated above).

**SDS-PAGE and immunoblotting.** Samples were prepared by boiling in standard denaturing and reducing SDS-PAGE sample buffer and separated using a 15% polyacrylamide gel. Lanes were equally loaded according to total protein content as determined by a Bradford assay. Proteins were visualized using Sypro ruby stain (Pierce) according to manufacturer protocols.

Gels were transferred to nitrocellulose for immunoblotting for a minimum of 30 min at 15 V. After blocking with Odyssey blocking buffer (Licor), primary antibody was added at a 1:5,000 dilution for 1 h to overnight at 4°C. Following membrane washing, Odyssey fluorescent antibody (goat anti-rabbit IRDye 800CW) was diluted to a working concentration of 1:20,000 according to manufacturer protocol. Immunoblots were analyzed with an Odyssey imaging system (Licor Biosciences).

**Electron microscopy.** In advance, 400-mesh copper grids with carbon films deposited on them (no. CF400-cu; Electron Microscopy Sciences) were cleaned via glow discharge for 1.5 min on a Harrick plasma cleaner (PDC-32G). Samples were prepared by applying 10  $\mu$ l to the grid and incubating for 2 min; grids were then washed with 5 drops of 1% aqueous uranyl acetate (Electron Microscopy Sciences). The last drop was left to incubate on the grid for 1.5 min before being wicked off with torn filter paper. Grids were left to dry for 5 min before being viewed on a Tecnai 12 by FEI with a 1,024-by-1,024 Gatan multiscan camera, model 794.

**LPS purification.** *P. aeruginosa* LPS was isolated by ethanol-Mg<sup>2+</sup> precipitation (46). Cell pellets were resuspended in 10 mM Tris-HCl (pH 8) buffer containing 2 mM MgCl<sub>2</sub>, DNase I, and RNase A and passed twice through a French pressure cell. To ensure complete cell breakage, the cell lysate was sonicated for two 30-s bursts. Samples were incubated at 37°C for 2 h with additional RNase A (50  $\mu$ g/ml) and DNase I (200  $\mu$ g/ml). Tetrasodium EDTA and SDS were added to reach a final volume of 25 ml and a concentration of 2% SDS–0.1 M tetrasodium EDTA, dissolved in 10 mM Tris-HCl (pH 8). Samples were vortexed to solubilize LPS. To remove peptidoglycan, the samples were centrifuged at 50,000  $\times$  g for 30 min at 20°C. The supernatant was decanted and incubated with pronase at 37°C overnight with shaking.

Two volumes of 0.375 M MgCl<sub>2</sub> in 95% ethanol were added and cooled to 0°C. After cooling, the samples were centrifuged at 12,000  $\times$  g for 15 min at 0 to 4°C. The pellet was resuspended in 2% SDS–0.1 M tetrasodium EDTA, dissolved in 10 mM Tris-HCl (pH 8) and sonicated to resuspend. Samples were incubated at 85°C for 10 to 30 min to denature protein. After cooling, pronase was added and incubated at 37°C overnight with shaking. After incubation, LPS was precipitated in 0.375 M MgCl<sub>2</sub> in 95% ethanol and pelleted as described above. Finally, the pellet was resuspended in 10 mM Tris-HCl (pH 8) and centrifuged at 200,000  $\times$  g for 2 h at 15°C in the presence of 25 mM MgCl<sub>2</sub>, and the LPS pellet was resuspended in distilled water.

**Statistical analysis.** All experimental data were performed with a minimum of three biological replicates. Statistical significance of data was determined by using a Student's *t* test. Raw data were combined across experimental trials. The averages for the untreated and treated cultures were compared to determine fold change. For experiments using the lipophilic dye FM4-64, the fluorescence of the probe alone in PBS was subtracted from all raw data. Immunoblot densitometry values consist of the integrated intensity of the gel band or area highlighted as determined with Licor Odyssey software. Densitometry of SDS-PAGE bands/lanes was performed with ImageJ. Error bars in the figures represent standard errors.

## RESULTS

**OMV production increases upon physiological stress.** Based on our previous studies in *E. coli*, we hypothesized that *P. aeruginosa* would respond to envelope stress with increased OMV production. The peptidoglycan synthesis inhibitor D-cycloserine functions as a chemical mimic for D-alanine, an amino acid that is required for peptidoglycan formation and bacterial outer membrane anchoring. In addition to perturbing cell wall assembly, D-cycloserine has an indirect effect that results in envelope stress because it also causes AlgU activation in *P. aeruginosa* PAO1 (24). AlgU in *P. aeruginosa* is the homologue of RpoE in *E. coli* (23, 24, 39).

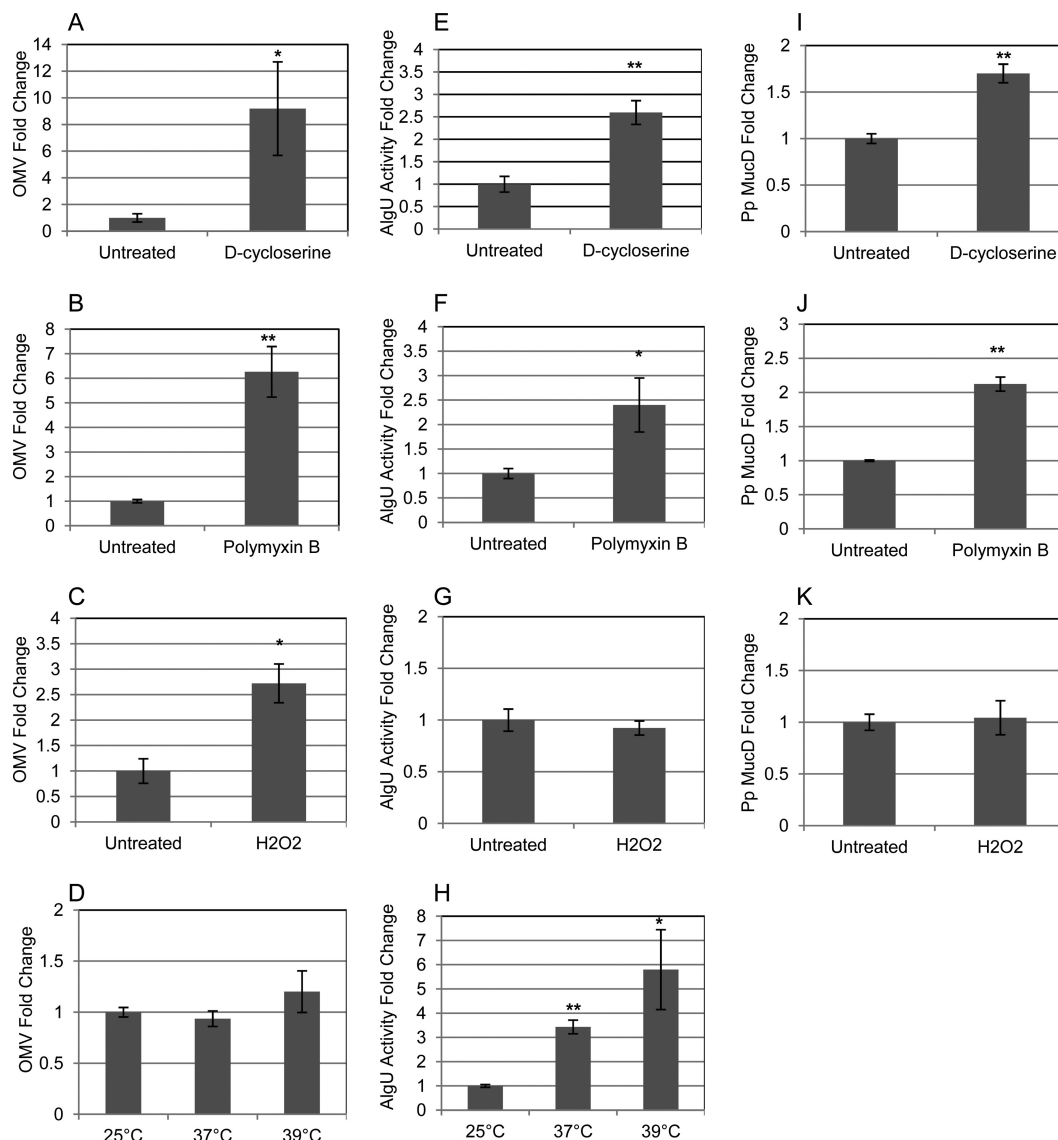
The amount of OMVs in the cell-free supernatant produced after the shift to the stress condition was quantified using FM4-64. FM4-64 is a dye that fluoresces upon incorporation into a lipid environment and was previously determined to report on the amount of OMVs in a cell-free culture supernatant (4, 8, 47). The other means of quantitating OMVs, measuring outer membrane protein content in the cell-free supernatant, was not feasible to use in these experiments, as stress conditions altered the protein com-

position of the membrane. By comparing the quantity of OMVs in the supernatants of untreated and D-cycloserine-treated late-log-phase cultures of *P. aeruginosa* PA14, we determined that vesiculation increased 9.2-fold (Fig. 1A). Bacterial viability following D-cycloserine treatment was compared with untreated preparations and the bacterial cell counts were not significantly different (see Table S1 in the supplemental material), suggesting that cell lysis did not account for the observed difference in yield. These data demonstrate that D-cycloserine treatment significantly induced production of OMVs.

To determine if physiologically relevant environmental stresses also stimulate OMV production by *P. aeruginosa*, we quantitated OMV levels after treatment with subinhibitory doses of three distinct stressors: hydrogen peroxide, increased temperature, and polymyxin B. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) treatment mimics the reactive oxygen burst that is produced by neutrophils against bacteria during infections. Temperature shifts from 25°C to 37°C and from 25°C to 39°C are experienced by bacteria during colonization from the environment into a host and particularly under febrile conditions in an acute infection. The antimicrobial peptide polymyxin B, which targets the bacterial outer membrane, is a clinically relevant chemotherapeutic stressor encountered by *P. aeruginosa* during clinical treatment of infections (48) and also mimics defensins produced by human tissues in response to infection (49).

Like D-cycloserine, treatments with 4  $\mu$ g/ml polymyxin B and 250  $\mu$ M H<sub>2</sub>O<sub>2</sub> significantly increased OMV production 6.3-fold and 2.7-fold, respectively (Fig. 1B and C). In contrast, neither a 12°C nor a 14°C rise in culture temperature caused a significant increase in OMV production (Fig. 1D). Bacterial viability following hydrogen peroxide and temperature treatments was compared with that in untreated preparations, and bacterial cell counts were not significantly different (see Table S1 in the supplemental material). Polymyxin B treatment did result in significantly fewer cells (2.3-fold) than were seen in untreated preparations; therefore, we needed to check that lysed cells did not account for the increased material in the OMV pellets. The presence of lysis products in the OMV-containing pellets would have yielded a protein profile different from OMVs, yet the protein profiles of the pelleted supernatant from untreated and treated cultures were not significantly different (see Fig. S1A in the supplemental material). From these data, we concluded that both hydrogen peroxide and polymyxin B treatments increased OMV formation, similar to the addition of D-cycloserine, whereas an increase in temperature did not result in an increase in OMV production over the course of the experiment.

**Stress-induced vesicle production is independent of AlgU activation.** The alternative sigma factor  $\sigma^E$  (RpoE) is responsible for the activation of the  $\sigma^E$  envelope stress pathway in *E. coli*, and overexpression of RpoE resulted in high levels of vesiculation in *E. coli* and *Salmonella enterica* serovar Typhimurium (our unpublished data). We monitored an AlgU-dependent promoter using a LacZ reporter and determined that, consistent with prior reports (50), treatment with 250  $\mu$ g/ml D-cycloserine increased AlgU activity (Fig. 1E). These data demonstrate that D-cycloserine stress significantly induced production of OMVs concurrently with AlgU activation. We tested whether there was also a direct correlation between stress-induced vesiculation and activation of AlgU for the other stress treatments. Polymyxin B and the 12°C and 14°C temperature shifts all increased AlgU activity (2.4-, 3.4-, and

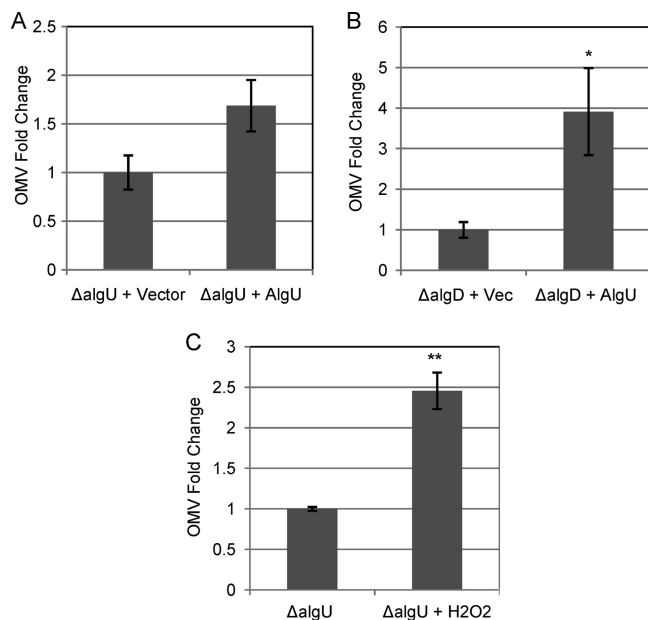


**FIG 1** Treatments with stressors induce OMV production in PA14 independent of AlgU and MucD levels. OMVs were collected and quantitated from cultures of PA14/pLW127 treated with 250  $\mu$ g/ml D-cycloserine (A), 4  $\mu$ g/ml polymyxin B (B), or 250  $\mu$ M hydrogen peroxide ( $H_2O_2$ ) (C). OMV yields were averaged and normalized to untreated controls (untreated) to calculate fold change. (D) OMVs were collected and quantitated from cultures of PA14 pLW127 grown at 25°C and shifted to 37°C or 39°C. OMV yields were averaged and normalized to a culture maintained at 25°C (25°C). AlgU promoter activity was measured in cultures (37°C; OD<sub>600</sub>, 0.9 to 1.1) of PA14/pLW127 treated with 250  $\mu$ g/ml D-cycloserine (E), 4  $\mu$ g/ml polymyxin B (F), or 250  $\mu$ M hydrogen peroxide ( $H_2O_2$ ) (G) using a  $\beta$ -galactosidase assay. Values were averaged and normalized to untreated controls (untreated) to calculate fold change. (H) AlgU promoter activity was measured in cultures (OD<sub>600</sub>, 0.9 to 1.1) of PA14/pLW127 grown at 25°C and shifted to 37°C or 39°C. Values were averaged and normalized to a culture maintained at 25°C to calculate fold change. Periplasmic MucD expression in PA14/pLW127 15 to 30 min after treatment with or without 250  $\mu$ g/ml D-cycloserine (I), 4  $\mu$ g/ml polymyxin B (J), or 250  $\mu$ M hydrogen peroxide ( $H_2O_2$ ) (K) was determined by densitometry. Values were averaged and normalized to the untreated samples to calculate fold change. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$  ( $n \geq 3$ ).

5.8-fold, respectively) (Fig. 1F and H). The temperature shift results were surprising, as OMV production did not increase concurrently with the increase in AlgU expression. Also quite unexpectedly, hydrogen peroxide treatment did not result in increased AlgU activity despite causing increased vesiculation (Fig. 1G). From these data we concluded that OMV production is independent of AlgU activation for some environmental stressors.

**AlgU activation is sufficient but not necessary to induce OMV formation.** In order to test directly whether there is a relationship between AlgU activation and outer membrane vesicula-

tion, we used strain PA14  $\Delta$ algU/pIM001, in which the only copy of *algU* exists on an inducible expression plasmid. We induced expression of AlgU during mid-log phase and quantitated OMVs once the cultures reached late log phase. We found that AlgU overexpression did not correlate with a significant increase in OMV production, although some increase was noted (Fig. 2A). Since  $\Delta$ algU mutants have a higher basal level of OMV production and overexpress alginate, which could interfere with OMV purification (43, 51), we also tested the overexpression of AlgU in PA14  $\Delta$ algD. *algD* encodes GDP-mannose dehydrogenase and is



**FIG 2** AlgU expression in PA14 is sufficient but not necessary for OMV production. (A) OMVs were collected and quantitated from cultures of PA14  $\Delta algU$ /pMF54 ( $\Delta algU$  + vector) and PA14  $\Delta algU$ /pIM001 ( $\Delta algU$  + AlgU) that had both been supplemented with 1 mM IPTG at mid-log phase. The difference was not significant ( $P = 0.068$ ). (B) OMVs were collected and quantitated from cultures of PA14  $\Delta algD$ /pMF54 ( $\Delta algD$  + vector) and PA14  $\Delta algD$ /pIM001 ( $\Delta algD$  + AlgU) that had both been supplemented with 200  $\mu$ M IPTG at mid-log phase. (C) OMVs were collected and quantitated from cultures of PA14  $\Delta algU$  ( $\Delta algU$ ) treated with 250  $\mu$ M hydrogen peroxide ( $\Delta algU$  + H<sub>2</sub>O<sub>2</sub>). OMV yield was normalized to that of the vector-containing (A and B) or untreated culture (C) to calculate fold change. \*,  $P < 0.05$ ; \*\*,  $P \leq 0.01$  ( $n = 3$ ).

the first gene in the alginate biosynthesis operon (52). Deletion of *algD* prevents synthesis of alginate but does not affect the envelope stress response functions of AlgU. OMVs were quantitated following mid-log-phase induction, and we found that increased levels of AlgU could significantly increase OMV production in the  $\Delta algD$  background (Fig. 2B). These results indicated that, as with RpoE in *E. coli*, increased levels of AlgU are sufficient to stimulate outer membrane vesiculation in *P. aeruginosa* but that the extent by which AlgU stimulates OMV production depends on the background strain.

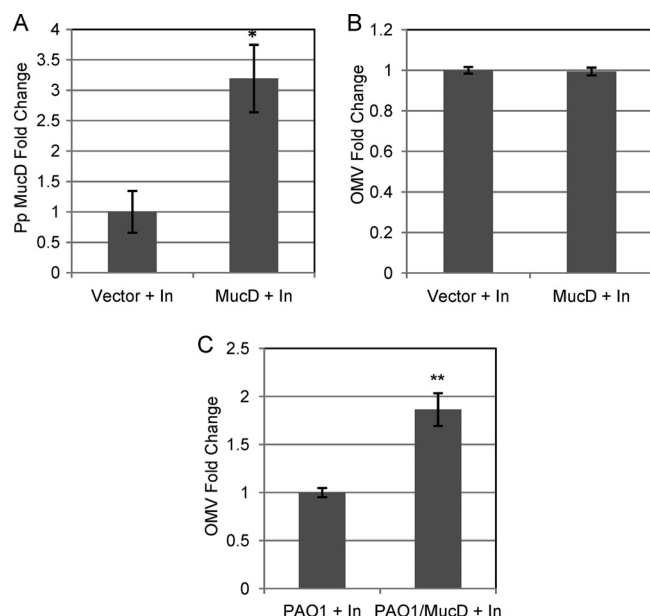
We next examined the role of AlgU in stress-induced vesicle production using a PA14  $\Delta algU$  strain. Experiments to test  $\sigma^E$ -dependent vesiculation cannot be performed in *E. coli*, due to *rpoE* being an essential gene. The  $\Delta algU$  mutation significantly increased the constitutive levels of vesiculation by PA14, confirming a previous report (43) (data not shown). We tried studying the effects of polymyxin B and D-cycloserine on OMV production in PA14  $\Delta algU$ ; however, this strain was hypersensitive to these agents, and the experiment could not be interpreted. Since PA14  $\Delta algU$  was not hypersensitive to oxidative stress, we were able to determine if oxidation-induced vesiculation was AlgU dependent. We found that PA14  $\Delta algU$  increased significantly with hydrogen peroxide treatment (2.5-fold) (Fig. 2C), similar to the increase observed using a WT background (Fig. 1C). These results indicate that AlgU activation is not necessary for the induction of OMV production in response to oxidative stress.

**Levels of periplasmic MucD are not inversely related to OMV production during stress.** Tashiro et al. concluded that as an extension of the AlgU envelope stress response mechanism for outer membrane vesiculation, OMV production is inversely related to the amount of the periplasmic chaperone MucD (43). It was suggested that low levels of MucD result in accumulation of misfolded protein in the periplasmic space, promoting OMV formation, whereas high levels of MucD result in low levels of protein accumulation, decreasing OMV production. If this MucD-dependent model holds for stress-induced vesiculation, then we would predict that the increased OMV formation we observed upon treatment with polymyxin B, D-cycloserine, and hydrogen peroxide should correlate with decreased levels of MucD in the periplasm.

To test whether stress-induced vesiculation is MucD level-dependent, we measured the amount of MucD in the periplasm of untreated and treated *P. aeruginosa* PA14. Cultures were exposed to stressors for only 15 to 30 min prior to isolating periplasmic content so that we could assess immediate changes in MucD levels upon stress. Equivalent amounts of total periplasmic protein were then separated using an SDS-PAGE gel, and MucD was detected by immunoblotting (see Fig. S2 in the supplemental material). As quantified by densitometry, periplasmic levels of MucD increased upon addition of D-cycloserine and polymyxin B (Fig. 1I and J). Thus, these cells exhibited elevated levels of MucD and elevated levels of OMV formation upon cell envelope-directed stress, a result which was not predicted from the proposed model. In addition, treatment with 250  $\mu$ M hydrogen peroxide did not alter the expression of MucD in the periplasm, despite increasing OMV production (Fig. 1K).

We also tested the consequence of MucD overexpression in unstressed conditions. Upon substantial induction of MucD expression (Fig. 3A; also, see Fig. S3 in the supplemental material), no change in OMV production was noted in the PA14 background (Fig. 3B), although overexpression of MucD in PAO1 resulted in a 1.9-fold increase in OMV production (Fig. 3C). These data suggest that strain-dependent differences could lead to different amounts of OMV production in response to MucD overexpression. Nevertheless, together, these results demonstrate that an increase in MucD expression does not necessarily result in a decrease in OMV production and that high OMV production does not necessarily result from decreased MucD expression.

**Oxidative stress-induced vesiculation cannot be rescued by MucD overexpression.** As described above, hydrogen peroxide-induced oxidative stress increased OMV production. Since oxidative damage might cause the accumulation of misfolded MucD substrates in the periplasm, we wondered if increasing MucD expression could suppress the oxidative stress response. We tested this hypothesis by examining OMV formation in cultures stressed with hydrogen peroxide and induced for MucD expression. We found that induction of MucD expression in cultures treated with hydrogen peroxide resulted in a significant increase in OMVs beyond that induced by peroxide alone (Fig. 4). No significant differences were detected in the protein profiles for the vector and MucD overexpression OMV preparations (see Fig. S1B in the supplemental material). As discussed previously, the similarity of these protein profiles showed that cell lysis was unlikely to contribute to the increase in OMV quantitation despite the 2-fold difference in CFU observed between the two treatments (see Table S1 in the supplemental material). We conclude that overexpres-

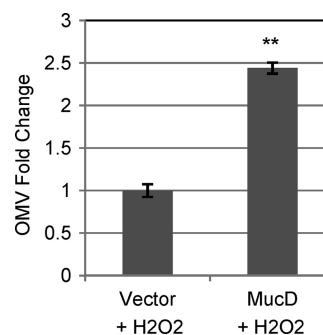


**FIG 3** Overexpression of MucD increases OMV production by PAO1, but not PA14. (A) Periplasmic MucD expression in cultures of PA14/pMF54 (Vector + In) or PA14/pLW112 (MucD + In) induced with 1 mM IPTG was measured by densitometry of immunoblotted samples and the values normalized to the vector control to calculate fold change. (B) OMVs were collected and quantitated from cultures of PA14/pMF54 (Vector + In) and PA14/pLW112 (MucD + In) both induced with IPTG at mid-log phase, and OMV yield was normalized to the induced vector control (Vector) to calculate fold change. (C) OMVs were collected and quantitated from cultures of PAO1/pMF54 (PAO1 + In) and PAO1/pLW112 (PAO1/MucD + In) both induced with IPTG at mid-log phase, and OMV yield normalized to the induced vector control (PAO1 + In) to calculate fold change. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$  ( $n = 3$ ).

sion of MucD cannot alleviate the stress caused by oxidative damage and that, instead, MucD overexpression can contribute to an additional increase in OMV production under hydrogen peroxide stress.

**Constitutive and stress-induced vesiculation is independent of PQS.** The quorum-sensing molecule PQS has been demonstrated to increase outer membrane blebbing of *P. aeruginosa* by interacting with molecules of outer membrane LPS and was proposed to be “required and sufficient” for vesicle formation in *P. aeruginosa* (36–38). Despite these reports, we found that the  $\Delta pqsA$  mutant strain, which is unable to synthesize the precursor molecules in the biosynthesis pathway of PQS, produced sufficient levels of OMVs to quantitate in our assays. The bacterial cell-free supernatant from WT and  $\Delta pqsA$  cultures at late log phase of growth were concentrated using ammonium sulfate precipitation, and OMV-containing fractions were pooled from a density gradient. We found not only that the  $\Delta pqsA$  strain constitutively produced OMVs but also that OMV production normalized to CFU for  $\Delta pqsA$  was significantly greater than that of PA14, ~3-fold as determined by FM4-64 (Fig. 5A) and ~2-fold by outer membrane protein densitometry (see Fig. S4A and B in the supplemental material). The presence of OMVs was also observed in negative stained electron microscopy images (see Fig. S4C and D).

Our results for OMV production by  $\Delta pqsA$  were surprising, as they differed significantly from previously published results. We considered whether this might be due to differences in the growth phase of the assayed cultures, in the OMV isolation and quantita-

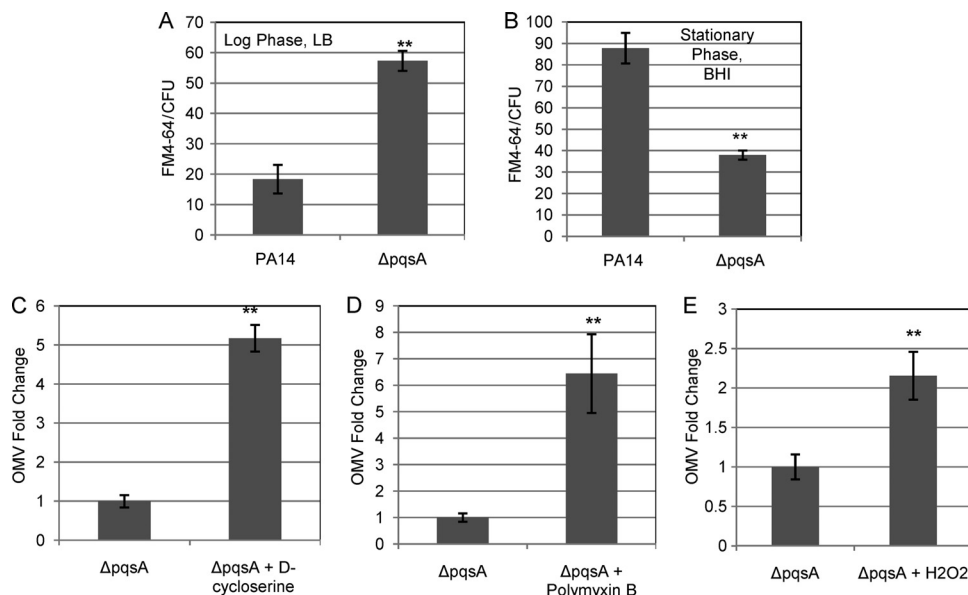


**FIG 4** MucD overexpression does not decrease oxidative stress induction of OMVs in PA14. OMVs were collected and quantitated from cultures of PA14/pMF54 (Vector + H2O2) and PA14/pLW112 (MucD + H2O2), both induced with IPTG and treated with 250  $\mu$ M hydrogen peroxide at mid-log phase. OMV yield was normalized to the induced vector control (Vector + H2O2) to calculate fold change. \*\*,  $P \leq 0.01$  ( $n = 3$ ).

tion methods, or both. We had assessed OMV production in log-phase LB cultures, whereas the previous studies used overnight BHI cultures. In addition, we had isolated density-purified OMVs from cell-free supernatant and quantitated them using FM4-64, whereas the prior study used absorbance at 220 nm of material pelleted from the cell-free supernatant. When we used the culture conditions, preparative methodologies, and an  $A_{220}$  quantitation method similar to that used in the prior study, we also observed a significant decrease in material pelleted from the  $\Delta pqsA$  cell-free supernatant compared to the wild type, although this decrease was approximately 2-fold, which was not as substantial as expected (data not shown). Similarly, using FM4-64, we detected 57% less material pelleted from the cell-free culture supernatant from the PA14  $\Delta pqsA$  cultures compared to the wild-type (Fig. 5B). Densitometry of OMV protein bands could not be used because pelleted supernatants contain proteins other than those typically seen in density gradient-purified OMVs (9). From these data we concluded that OMVs were constitutively produced by PA14 during both log and stationary phases of growth without a requirement for the quorum-sensing molecule PQS; however, we noted a difference in OMV production for early and late growth phases that was PQS dependent.

To finally test whether OMV induction upon stress treatment was PQS dependent, cultures of PA14  $\Delta pqsA$  were treated with stressors and OMVs were quantitated. The addition of D-cycloserine, polymyxin B, and hydrogen peroxide treatments to PA14  $\Delta pqsA$  increased OMV production 5.2-, 6.4-, and 2.2-fold, respectively (Fig. 5C to E), similar to the increases seen with the WT strain (Fig. 1A to C). Therefore, stress-induced OMV production by *P. aeruginosa* does not require either the production or the presence of PQS in the culture.

**Oxidative stress-induced vesicle production is B-band LPS dependent.** *P. aeruginosa* outer membrane LPS is composed of both B- and A-band subtypes. Previously, it was demonstrated that increased levels of oxygen resulted in increased B-band LPS compared to the amount produced in an anaerobic environment and that levels of OMV formation increased with increased levels of saturated oxygen (53). Therefore, we wanted to investigate whether B-band LPS was required for the induction of OMVs by hydrogen peroxide stress. We used LPS mutants which are unable to synthesize A-band (PA14  $\Delta rmd$ ), B-band (PA14  $\Delta wbpM$ ), or



**FIG 5** PQS is not required for constitutive or stress-induced OMV production in PA14. (A) PA14 and PA14  $\Delta pqsA$  were grown to late log phase in LB broth, and the cell-free supernatants were precipitated using ammonium sulfate. OMVs were purified from the concentrated supernatant by density gradient fractionation. OMV production was determined by FM4-64 quantitation of lipid in the OMV fractions (FM4-64/CFU). (B) PA14 and PA14  $\Delta pqsA$  were grown overnight in BHI broth and the cell-free supernatants were ultracentrifuged to isolate OMVs. OMV production was determined by FM4-64 quantitation of lipid in the OMV fractions (FM4-64/CFU). (C to E) OMVs were collected and quantitated from cultures of PA14  $\Delta pqsA$  treated with 250  $\mu\text{g/ml}$  D-cycloserine (C), 4  $\mu\text{g/ml}$  polymyxin B (D), or 1 mM hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (E). OMV yield was normalized to untreated controls ( $\Delta pqsA$ ) to calculate fold change. \*\*,  $P \leq 0.01$  ( $n \geq 3$ ).

both A- and B-band (PA14  $\Delta wbpR$ ) LPS (54). Differences in LPS expression were confirmed by silver staining (see Fig. 5 in the supplemental material). We found that hydrogen peroxide treatment reduced OMV production of the B-band mutant ( $\Delta wbpM$ ) by >60%, whereas peroxide stress induced the A-band mutant ( $\Delta rmd$ ) to produce ~7-fold more OMVs (Fig. 6). The basal level of vesicle production by the A- and B-band double mutant ( $\Delta wbpR$ ) was 2-fold above that by PA14, but the addition of oxidative stress did not result in a further increase in OMV production (Fig. 6). Together, the data suggest that hydrogen peroxide-

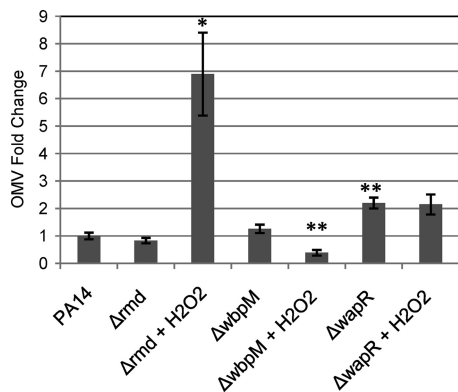
induced OMV production depends on the presence of B-band but not A-band LPS.

## DISCUSSION

Gram-negative bacteria must successfully react to environmental stressors in order to survive. For pathogenic bacteria, the hostile host environment is a critical challenge to overcome during the establishment of infection. We propose here that OMVs are induced by *P. aeruginosa* in response to physiological stressors, such as those encountered during infection, and that the mechanism of stress-induced OMV production is independent of known modulators.

We first examined the relationship between environmental stressors, AlgU levels, and induction of OMV production. The  $\sigma^E$  envelope stress response pathway in *E. coli* is activated upon sensing misfolded outer membrane proteins in the periplasmic space (39). Based on homology and conservation of this stress response pathway in *P. aeruginosa* (23, 24, 39), it can be inferred that the AlgU-mediated response functions in a similar manner. These homologous pathways provide a means for the bacteria to manage accumulated misfolded proteins in the periplasm as a result of environmental stress.

Treatment with cell wall-directed compounds, polymyxin B and D-cycloserine, resulted in an increase in OMV production with a concomitant (and previously reported for D-cycloserine [24]) increase in AlgU activity. Similar responses to these treatments were expected because both compounds physically perturb the outer membrane. Although the hypersensitivity of  $\Delta algU$  to these stressors prevented us from determining if AlgU was critical to the induction of OMVs by polymyxin B or D-cycloserine, it was noteworthy that the  $\Delta algU$  strain was hypersensitive. AlgU ex-



**FIG 6** B-band LPS is required for  $\text{H}_2\text{O}_2$ -induced OMV formation in PA14. OMVs were collected and quantitated from untreated cultures of PA14, PA14  $\Delta wbpM$  (B-band mutant), PA14  $\Delta rmd$  (A-band mutant), and PA14  $\Delta wbpR$  (A- and B-band mutant) and from cultures treated with 250  $\mu\text{M}$  hydrogen peroxide ( $\Delta wbpM + \text{H}_2\text{O}_2$ ,  $\Delta rmd + \text{H}_2\text{O}_2$ ,  $\Delta wbpR + \text{H}_2\text{O}_2$ ). OMV yield was normalized to that of an untreated culture of PA14 (PA14). \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$  ( $n \geq 3$ ).

pression typically results in increased alginate production which provides the bacterium protection against phagocytosis, oxidative stress, and complement (55). Thus, the  $\Delta algU$  hypersensitive phenotype suggests that the activation and function of AlgU plays an additional, more general, protective role against agents that physically disrupt the cell wall. Additional stability may be mediated by the AlgU-controlled periplasmic MucD chaperone. In response to envelope stress, increased expression of MucD could help maintain the functionality of the periplasm as well as the membrane architecture.

Temperature change was examined as a general stressor that *P. aeruginosa* typically encounters during the course of infection. Shifting *P. aeruginosa* from growth at 25°C to 37°C or 39°C mimics the change in environment that the pathogen would encounter upon moving from the external environment into a human host. Increasing the temperature did not result in an increase in OMV production but did increase the amount of AlgU activity. The increase in AlgU is consistent with previous reports that AlgU is regulated by temperature and leads to the regulation of heat shock genes such as *rpoH* (56, 57). The fact that OMV production did not change, despite the change in AlgU, was surprising. However, this result may be explained if a threshold level of AlgU activation is needed to induce OMV production and this level was not achieved in the experiments. Indeed, heat shock genes are commonly expressed at temperatures higher than our experimental design. Nevertheless, the data demonstrate that there is not a linear correlation between AlgU activation and OMV production in *P. aeruginosa*.

Oxidative stress was tested as another general stressor that *P. aeruginosa* would encounter during the course of infection, and we determined that oxidative stress resulted in a significant increase in OMV production. Reactive oxygen species such as hydrogen peroxide are produced by innate immune cells and released upon contacting an infectious agent. Reactive oxygen is a very potent defense mechanism because it targets all aspects of the cellular machinery, and bacteria cannot evolve a single mechanism to evade reactive oxygen species. Because overexpression of MucD could not mitigate peroxide-induced production of OMVs, the data suggest that oxidative stress is unlikely to result in the accumulation of misfolded periplasmic protein. Consistent with this, unlike either the responses to cell wall or temperature stressors, oxidative stress did not result in increased AlgU activity.  $\Delta algU$  cells were not hypersensitive to oxidative stress; therefore, we were able to address directly whether induction of OMV production depended on AlgU. Notably, these results demonstrated that AlgU was not necessary to produce constitutive or stress-induced levels of OMVs in *P. aeruginosa*.

Despite AlgU not being required for OMV production, overexpression of AlgU did prove to be sufficient to increase OMV production. This result was similar to our previous studies of RpoE in *E. coli*. The increase in OMV production in *P. aeruginosa* and *E. coli* due to overexpression of AlgU or RpoE, respectively, is possibly indirect, due to the downstream effects of changes in periplasmic chaperone and envelope component expression that cause crowding and or misfolding of envelope proteins.

The quorum-sensing molecule PQS was proposed to be critical for OMV formation due to the secretion phenotype of a *P. aeruginosa* mutant lacking PQS (PA14  $\Delta pqsA$ ) (36). We confirmed a decrease in basal levels of OMV production by the mutant when cultures were grown overnight and yield was quantitated using

previously reported methods; however, we observed a surprising increase in OMV production for log-phase cultures. In any event, we reasoned that PQS was unlikely to be responsible for the increase in OMV formation we had observed upon stress, since our cultures were examined during log phase, when PQS is not highly expressed. PQS, as a quorum-sensing molecule, is expressed during late log phase and via the LasR/LasI and RhIR/RhII quorum-sensing systems (58, 59). Indeed, we observed increases in OMV production for untreated as well as D-cycloserine-, polymyxin B-, and hydrogen peroxide-treated log-phase cultures despite the absence of PQS. These data confirm that PQS does not represent the sole driving force for OMV formation in *P. aeruginosa* and suggest a growth phase-dependent regulation of OMV production by PQS.

Since hydrogen peroxide stress elicited AlgU- and PQS-independent OMV induction, we were prompted to test another proposed regulatory mechanism for OMV formation in *P. aeruginosa*. Recently it was proposed that OMV production in *P. aeruginosa* inversely correlates with the level of the periplasmic chaperone, MucD. However, we found that upon addition of hydrogen peroxide treatment, MucD levels remained unchanged despite an increase in OMV production. If MucD levels and OMV production were inversely proportional, MucD levels would have been decreased in treated cells. In addition, we observed that cell envelope-directed stressors which caused elevated OMV and AlgU production also resulted in an increase in MucD, which is again contrary to the proposed model. This result is, in fact, consistent with that previously reported for *E. coli*, which indicated that overexpression of the MucD homolog, DegP, resulted in an increase in OMV production (12).

In the course of studying the relationship between MucD levels and OMV induction in stressed cultures, we also tested unstressed cultures. We observed no decrease and an increase in OMV production upon MucD overexpression in PA14 and PAO1 backgrounds, respectively. This was notable, since it was previously reported that MucD overexpression in the *P. aeruginosa* strain PAO1 resulted in decreased OMV production. Differences in expression levels and plasmid copy numbers in the different strains and studies may provide a rationale for this discrepancy. It is possible that a low level of expression of MucD may reduce any accumulated periplasmic stress, resulting in a decrease in OMV production, whereas a higher level of expression results in MucD becoming an accumulated protein itself. As a result of all these data, we conclude that additional mechanisms beyond MucD- or PQS-mediated processes must exist that control OMV production in *P. aeruginosa*.

OMV production in *P. aeruginosa* has been linked to the presence of B-band LPS in two prior studies. Natively produced OMVs from WT cells were reported to contain only B-band LPS, whereas the addition of cell envelope-acting compounds to the cultures resulted in the presence of both A- and B-band LPS in the OMVs (13). Also, increased levels of saturated oxygen resulted in an increase in cellular B-band LPS as well as in OMV production (53). We therefore tested whether B-band LPS was required for the increase in induced OMV production using the physiological stressor hydrogen peroxide. Our finding that B-band LPS is critical for *P. aeruginosa* to produce OMVs in response to oxidative stress could explain why *P. aeruginosa* strains lacking O antigen were reported to be more susceptible to oxidative stresses (60).

In conclusion, the activation of pathways that regulate OMV

formation in *P. aeruginosa* is coincident with activation of the numerous pathways that manage the variety of environmental stresses that an organism faces upon entering a host, making it challenging to single out their effects. Nevertheless, this study demonstrates that environmental stresses result in increased OMV formation by *P. aeruginosa* that are independent of the previously identified modulators of OMV production, MucD and PQS, as well as the homolog of a modulator of OMV production in *E. coli*, AlgU. Further, we confirmed that B-band but not A-band LPS was required for oxidative stress-induced OMV production. How the induction of OMVs helps *P. aeruginosa* to cope with the stressful environment of the host warrants future investigation.

## ACKNOWLEDGMENTS

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